Novel Injection Method to Reduce Dedifferentiation of Chondrocytes Cultured in Alginate Scaffolds

Layla Barkal, Dahlia Alkekhia, 20.109 teaching faculty

Introduction:

Cartilage is a tissue that is difficult to regenerate, frequently damaged, and that causes pain in millions of people every year. Tissue engineering provides the tools with which to solve this problem, the tools that may develop a therapy to help those suffering from damaged cartilage. The current issues surrounding regeneration of cartilage tissue are primarily concerned with dedifferentiation of cartilage cells grown in culture. If dedifferentiation of cartilage cells in culture could be reduced, it may be possible to develop a method to grow cartilage tissue *in vitro* that could then be used therapeutically.

Current techniques of growing tissue primarily involve the use of scaffolds. Alginate beads are commonly used as cell scaffolds, though their ability to keep cartilage cells differentiated is limited. The cells are mixed with the alginate prior to polymerization and therefore the cells are trapped within beads after polymerization. This method of inserting the cells into the beads, though, lacks enough control to predict the amount of contact cells will have with other cells. It has been shown that cell contact plays an important role in dedifferentiation (Echeverri 2002 and Weinberg 2007) and therefore, our developed method preserved the alginate bead scaffold while increasing contact between cells; after polymerization of the alginate beads, a cell solution was inserted within the bead. After being cultured (as per the online protocol) the levels of collagen types I and II were measured in order to determine whether or not the cells cultured with more cell contacts had reduced levels of dedifferentiation; in chondrocytes, as opposed to other cell types, the level of collagen type II should be much higher than that of collagen type I.

Results:

To create the alginate scaffolds, sigma 1% alginate droplets were put into calcium chloride, a treatment which caused their polymerization. The control sample used alginate that already had 1e6 cells mixed in with it; the experimental sample used pure alginate. The experimental alginate beads were then injected using a syringe with 20uL cell solution (1e6 cells suspended in 200uL media) and both samples were cultured identically. To maintain the same number of cells in both the control and experimental samples, not all of the experimental alginate beads were able to be injected with cell solution.

Throughout the experiment, there were three samples that were analyzed: the control cells, those cells harvested from beads injected with cell solution, and cells gathered from the monolayer of cells escaped from the beads that grew on the bottom of the injected bead plate. To quantify the levels of dedifferentiation between the three samples, the levels of collagen types I and II were analyzed. Importantly, collagen type II is specific to chondrocyte cells while collagen type I is found primarily in cells other than chondrocytes. Therefore, a higher ratio of collagen type II : collagen type I in our experimental samples would indicate that there was decreased dedifferentiation of chondrocyte cells.

One of the ways we calculated this collagen ratio was using RT-PCR. For the RT-PCR test, RNA was isolated and 80ng of each sample was used. The RNA isolated from the experimental sample and that isolated from the monolayer cells both had relatively low A260/280 ratios indicating that the RNA was impure. RT-PCR was performed on the isolated RNA, each sample with a primer for GAPDH and either collagen type I or collagen type II. The resulting products were run on an agarose gel and imaged as can be seen in Figure 1. In all lanes, the lower band is GAPDH. The upper bands, corresponding to

collagen, were normalized with respect to the GAPDH bands in the same lanes and the collagen type II : collagen type I ratios calculated as can be seen in part B. The experimental collagen ratio of greater than one indicates that our experimental sample had more collagen type I and reduced levels of dedifferentiation in comparison with the control.

Another method of calculating the collagen type II to collagen type I ratio was using ELISA protein analysis. Primary antibodies against collagen types I and II were used in conjunction with a labeled secondary antibody. The samples were then developed as per the online protocol and analyzed using a plate reader at 420nm. By correcting for background fluorescence and normalizing the data, we were able to determine the approximate protein concentrations of our samples, specifically the amount of collagen types I and II present in the protein isolates. The ELISA results are displayed in Figure 2. The overall protein level for samples analyzed for both types of collagen was quite low. In the charts, the blue line corresponds to the standard samples against which our experimental samples were normalized and compared. The line of best fit was used to calculate the approximate level of collagen present in each sample. The collagen type II : collagen type I ratio was then calculated and is displayed in part C of Figure 2. The results do not match those determined from the RT-PCR analysis. Instead of showing a reduction in the amount of chondrocyte dedifferentiation, our experimentally injected alginate beads exhibit an increase in dedifferentiation.

Discussion:

One of the primary hurdles in creating a novel tissue re-growth therapy for cartilage loss is the inability of scientists to culture chondrocyte cells without their dedifferentiation. Through our novel design of an injection system we hoped to develop a method of *in vitro* cell culture that increased the

number of chondrocyte cells that remained differentiated. Unfortunately, the results of our cell viability assay were not useful and could not inform interpretation of our data; the injected beads were cultured with alginate beads that had not been injected and we therefore did not know if the beads we chose to assay had actually been injected with cell solution. Additionally, our control beads had a very low number of cells in the viability assay. To get more consistent and useful data, is important that this experiment be repeated with a larger number of beads and with a cell viability assay that gives useful information. In future experiments it will also be important to develop a method of injection that reduces the amount of cell solution that passes through the beads rather than staying in the center.

Overall, the results of our RT-PCR test are promising though our ELISA data contradicts these results. Therefore it is imperative that the experiment be repeated. Also, another analysis may be added to assay for chondrocyte dedifferentiation. Specifically, beads taken from the control and injected samples may be cut in half with one half being used for a cell viability assay and the other half used in ELISA with a proteoglycan target; the cell viability assay will confirm that cells are present on the sliced surface of the bead while the ELISA specific to proteoglycans, a protein secreted by chondrocyte cells, will show the relative amounts of differentiated chondrocytes present. Without repeating the experiment, it is impossible to say whether or not our novel injection method will improve the problem of chondrocytes dedifferentiation *in vitro*. If, after further study, it shown that our method is indeed successful, it will provide an important tool for tissue engineers to use in their endeavor to create cartilage tissue in culture that can be used as a therapeutic agent to help those suffering with damaged cartilage.

References:

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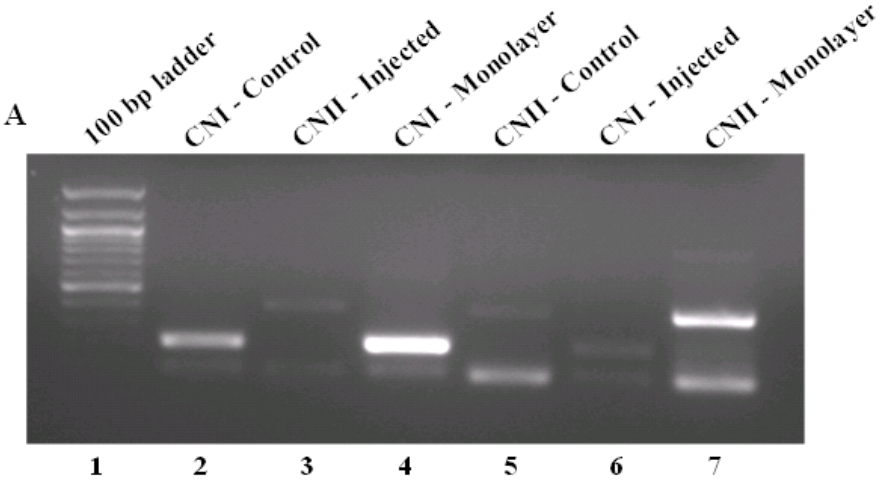
Karen Echeverri and Elly M. Tanaka. Mechanisms of muscle dedifferentiation during regeneration. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WX0-46T3XWD-6&_user=501045&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000022659&_version=1&_urlVersion=0&_userid=501045&md5=572d5705d28c07c679b82a1fae220b48

Figures:

A



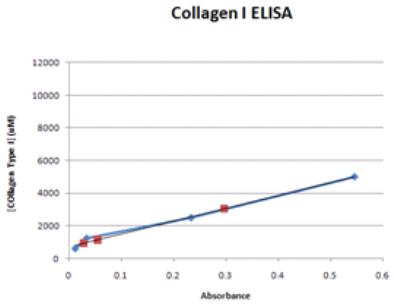
B

Collagen II : Collagen I Ratios

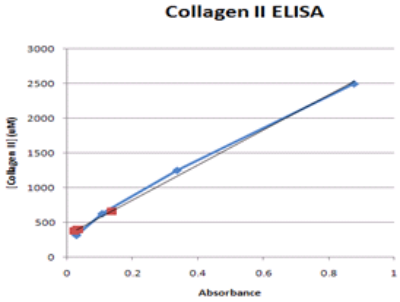
Control	0.043
Experimental	1.422
X-tra	0.331

Figure 1. RT-PCR results shows that injected beads have a higher collagen II / collagen I ratio than the control beads or monolayer cells. Part A shows RNA samples amplified using primers specific for GAPDH and either collagen type I or collagen type II run on an agarose gel and imaged for 8ms. The lower band in each column is GAPDH. All samples were normalized to GAPDH before the collagen ratios were calculated as displayed in part B.

A



B



C

Collagen II : Collagen I Ratios

Control	0.425
Experimental	0.330
X-tra	0.217

Figure 2. ELISA protein analysis indicates that all samples showed high levels of dedifferentiation with the control cells exhibiting the least. Protein was isolated from all three cell samples and treated with a primary antibody against either collagen type I or type II. They were then treated with a secondary antibody, developed, and analyzed for absorbance at 420nm. Parts A and B above show the known protein standards in blue and a line of best fit that was used to calculate the collagen levels present in the samples. Part C gives the ratio of collagen type II : collagen type I for all three samples. The results indicate that all samples had high levels of dedifferentiation with the control actually have the least.